



INVESTOR IN PEOPLE



The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

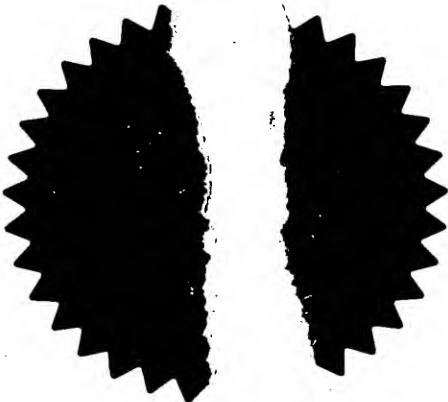
RECEIVED
MAR 19 2002
FRESH CENTER 1600/2900

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

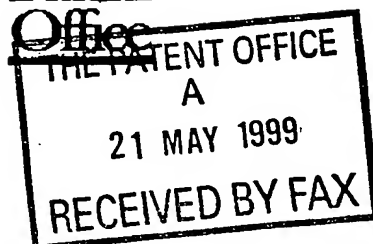


Signed

Dated

12 DEC 2001



21MAY99 E448944-1 C03046
P01/7700 0.00 - 9911812.7**Patents Form 1/77**Patents Act 1977
(Rule 16)The
Patent
Office**Request for a grant of a patent***(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)*

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference

P006915GBR CTH

2. Patent application number
*(The Patent Office will fill in this part)***9911812.7****21 MAY 1999**3. Full name, address and postcode of the or of each applicant
*(underline all surnames)*Oxford BioMedica (UK) Limited
Medawar Centre
Robert Robinson Avenue
The Oxford Science Park
Oxford
OX4 4GA
United KingdomPatents ADP number *(if you know it)*722352200/
United Kingdom

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Improved Retroviral Production

5. Name of your agent *(if you have one)*

D YOUNG & CO

"Address for service" in the United Kingdom to which all correspondence should be sent
*(including the postcode)*21 NEW FETTER LANE
LONDON
EC4A 1DAPatents ADP number *(if you have one)*

59006

6. If you are declaring priority from one or more earlier patent applications, give the country and date of filing of the or each of these earlier applications and (if you know it) the or each application number

Country

Priority application
number
*(if you know it)*Date of filing
(day/month/year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and filing date of the earlier application

Number of earlier
applicationDate of filing
(day/month/year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:
 a) any applicants named in part 3 is not an inventor, or
 b) there is an inventor who is not named as an applicant, or
 c) any named applicant is a corporate body.
 See note (d)) Yes

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form 0

Description 18

Claims(s) 2

Abstract 1

Drawing(s) 15

10. If you are also filing any of the following, state how many against each item.

Priority documents 0

Translations of priority documents 0

Statement of inventorship and right to grant of a patent (Patents Form 7/77) 0

Request for preliminary examination and search (Patents Form 9/77) 0

Request for substantive examination (Patents Form 10/77) 0

Any other documents No
 (please specify)

11. I/We request the grant of a patent on the basis of this application.

Signature

Date

Edith for D Young & Co
 D YOUNG & CO
 Agents for the Applicants

21.05.99

12. Name and daytime telephone number of the person to contact in the United Kingdom

Dr C T Harding

01703 634816

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 01645 500505

b) Write your answers in capital letters using black ink or you may type them

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

IMPROVED RETROVIRAL PRODUCTION

Field of the invention

- 5 The present invention relates to a method for improving the packaging efficiency of retroviral vectors.

Background to the invention

- 10 Retroviral technology has gained immense popularity in recent times for the stable delivery of genes into cells (for recent review, see Miller, 1997). The applications are widespread in the fields of medicine, where it is used to deliver therapeutic genes to rectify genetic disorders, and also in science generally, where it is used to introduce genes into cells so as to study their functions (Miller, 1997). One reason for the popularity of retroviruses is that
15 they are far more efficient in introducing genes to cells when compared to conventional methods of transfection. This is because the genes are packaged into virions which contain envelope proteins that bind to receptors on the target cells. This process enhances the entry of the gene into the cell.

- 20 Retroviruses are presented with a paradox in their life cycle. Interaction between the viral envelope and the cell receptor enables the virus to enter the cell. However, the same interaction between receptors in the infected cell and the newly synthesised envelope proteins limits the pool of envelope available for virion incorporation. In HIV, this problem is solved by the production of *vpu*, which down regulates the receptors on the
25 infected cell (Jabbar, 1995). In other retroviruses, it has been postulated that there is an overproduction of envelope proteins to down-regulate receptors on infected cells (Gilbert *et al.*, 1994).

Summary of the invention

30

We have found, while investigating using a three plasmid transient transfection method (Soneoka *et al.*, 1995) which components are limiting in retroviral production and under

-2-

P006915gb CTH

what conditions, that the envelope component is only limiting when its receptors are found in the 293T producer cells used.

Accordingly, to alleviate the limitation of envelope on viral production, it is an object of the present invention to down-regulate the receptors on producer cells so as to increase the amount of envelope available for incorporation into virions.

Thus, the present invention provides a method for enhancing the production of an infectious retrovirus in a producer cell which method comprises inhibiting the expression in the producer cell of an endogenous receptor which binds the envelope polypeptide of said retrovirus.

Preferably expression of the receptor is inhibited by expressing in the producer cell a gene product capable of binding to and effecting the cleavage, directly or indirectly, of a nucleotide sequence encoding the receptor, or a transcription product thereof.

Preferably the gene product is selected from a ribozyme, an anti-sense ribonucleic acid and an external guide sequence, more preferably a ribozyme.

In a preferred embodiment the infectious retroviruses produced by the producer cell are isolated for subsequent use.

The present invention also provides a composition comprising infectious retroviruses obtained by the method of the invention. Such compositions may be used in therapy.

The present invention further provides a method for producing a pharmaceutical composition which method comprises isolating the infectious retrovirus produced by the producer cell according to the method of the invention described above and admixing with a pharmaceutically acceptable carrier, diluent or excipient.

In a preferred embodiment, the present invention provide a nucleic acid comprising a nucleotide sequence encoding a ribozyme capable of binding to and effecting the cleavage of an RNA encoding a Pit2 receptor. Preferably, the nucleic acid comprises a nucleotide

sequence as shown in Figure 1 or a variant thereof capable of binding to and effecting the cleavage of an RNA encoding a Pit2 receptor.

Detailed description of the invention

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

Retroviruses

The retroviral vectors used in the production of infectious retroviruses according to the present invention may be derived from or may be derivable from any suitable retrovirus. A large number of different retroviruses have been identified. Examples include: murine leukemia virus (MLV), human immunodeficiency virus (HIV), simian immunodeficiency virus, human T-cell leukemia virus (HTLV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV). A detailed list of retroviruses may be found in Coffin *et al.*, 1997, "Retroviruses", Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763.

Details on the genomic structure of some retroviruses may be found in the art. By way of example, details on HIV and Mo-MLV may be found from the NCBI Genbank (Genome Accession Nos. AF033819 and AF033811, respectively).

The lentivirus group can be split even further into "primate" and "non-primate". Examples of primate lentiviruses include human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi

virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

5 The basic structure of a retrovirus genome is a 5' LTR and a 3' LTR, between or within which are located a packaging signal to enable the genome to be packaged, a primer binding site, integration sites to enable integration into a host cell genome and *gag*, *pol* and *env* genes encoding the packaging components - these are polypeptides required for the assembly of viral particles. More complex retroviruses have additional features, such as
10 *rev* and RRE sequences in HIV, which enable the efficient export of RNA transcripts of the integrated provirus from the nucleus to the cytoplasm of an infected target cell.

In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. LTRs also
15 serve as enhancer-promoter sequences and can control the expression of the viral genes. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral genome.

The LTRs themselves are identical sequences that can be divided into three elements,
20 which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

25 In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

In a typical retroviral vector for use in gene therapy, at least part of one or more of the *gag*,
30 *pol* and *env* protein coding regions essential for replication may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even be replaced by a nucleotide sequence of interest (NOI), such as a nucleotide sequence encoding a therapeutic product, to generate a virus capable of integrating its genome into a

-5-

P006915gb CTH

host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic and/or a diagnostic effect. Thus, the transfer of an NOI into a site of interest is typically achieved by: integrating the NOI into the recombinant viral vector; packaging the modified viral vector into a virion coat; and allowing transduction of a site of interest - such as a targeted cell or a targeted cell population.

A minimal retroviral genome for use in the present invention will therefore comprise (5') R - U5 - one or more first nucleotide sequences - U3-R (3'). However, the plasmid vector used to produce the retroviral genome within a host cell/packaging cell will also include transcriptional regulatory control sequences operably linked to the retroviral genome to direct transcription of the genome in a host cell/packaging cell. These regulatory sequences may be the natural sequences associated with the transcribed retroviral sequence, i.e. the 5' U3 region, or they may be a heterologous promoter such as another viral promoter, for example the CMV promoter.

Some retroviral genomes require additional sequences for efficient virus production. For example, in the case of HIV, *rev* and RRE sequence are preferably included. However the requirement for *rev* and RRE can be reduced or eliminated by codon optimisation.

Once the retroviral vector genome is integrated into the genome of its target cell as proviral DNA, the nucleotide sequences of interest need to be expressed. In a retrovirus, the promoter is located in the 5' LTR U3 region of the provirus. In retroviral vectors, the promoter driving expression of a therapeutic gene may be the native retroviral promoter in the 5' U3 region, or an alternative promoter engineered into the vector. The alternative promoter may physically replace the 5' U3 promoter native to the retrovirus, or it may be incorporated at a different place within the vector genome such as between the LTRs.

Thus, an NOI will also be operably linked to a transcriptional regulatory control sequence to allow transcription of the NOI to occur in the target cell. The control sequence will typically be active in mammalian cells. The control sequence may, for example, be a viral promoter such as the natural viral promoter or a CMV promoter or it may be a mammalian

-6-

P006915gb CTH

promoter. It is particularly preferred to use a promoter that is preferentially active in a particular cell type or tissue type in which the virus to be treated primarily infects. Thus, in one embodiment, a tissue-specific regulatory sequences may be used. The regulatory control sequences driving expression of the one or more first nucleotide sequences may be constitutive or regulated promoters.

Replication-defective retroviral vectors are typically propagated, for example to prepare suitable titres of the retroviral vector for subsequent transduction, by using a combination of a packaging or helper cell line and the recombinant vector. That is to say, that the three packaging proteins can be provided *in trans* (see below).

Producer cells

Retroviral producer cells are cells that contain all the elements necessary for the production of infectious recombinant retroviruses. These elements may be permanently present stably within the cell (for example integrated in the cell genome or in episomal form) and/or transiently provided, for example by transfection.

A packaging cell, by contrast, expresses one or more viral components required for packaging retroviral DNA but lacks a *psi* region. Packaging cell lines typically comprise one or more of the retroviral *gag*, *pol* and *env* genes. Thus, the packaging cell line produces the structural proteins required for packaging retroviral DNA but it cannot bring about encapsidation due to the lack of a *psi* region. However, when a recombinant vector carrying a defective viral genome comprising a *psi* region and typically a nucleotide sequence of interest (NOI) is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant vector to produce the recombinant virus stock. This virus stock can be used to transduce cells to introduce the NOI into the genome of the target cells. It is preferred to use a *psi* packaging signal, called *psi* plus, that contains additional sequences spanning from upstream of the splice donor to downstream of the *gag* start codon since this has been shown to increase viral titres.

The recombinant virus whose genome lacks all genes required to make viral proteins can transduce only once and cannot propagate. These viral vectors which are only capable of a

single round of transduction of target cells are known as replication defective vectors. Hence, the NOI is introduced into the host/target cell genome without the generation of potentially harmful retrovirus. A summary of the available packaging lines is presented in Coffin *et al.*, 1997.

5

Packaging cell lines in which the *gag*, *pol* and *env* viral coding regions are carried on separate expression plasmids that are independently transfected into a packaging cell line are preferably used. This strategy, sometimes referred to as the three plasmid transfection method (Soneoka *et al.*, 1995) reduces the potential for production of a replication-competent virus since three recombinant events are required for wild type viral production. As recombination is greatly facilitated by homology, reducing or eliminating homology between the genomes of the vector and the helper can also be used to reduce the problem of replication-competent helper virus production.

10

15

An alternative to stably transfected packaging cell lines is to use transiently transfected cell lines. Transient transfections may advantageously be used to measure levels of vector production when vectors are being developed. In this regard, transient transfection avoids the longer time required to generate stable vector-producing cell lines and may also be used if the vector or retroviral packaging components are toxic to cells. Components typically used to generate retroviral vectors include a plasmid encoding the *gag/pol* proteins, a plasmid encoding the *env* protein and a plasmid containing an NOI. Vector production involves transient transfection of one or more of these components into cells containing the other required components. If the vector encodes toxic genes or genes that interfere with the replication of the host cell, such as inhibitors of the cell cycle or genes that induce apoptosis, it may be difficult to generate stable vector-producing cell lines, but transient transfection can be used to produce the vector before the cells die. Also, cell lines have been developed using transient transfection that produce vector titre levels that are comparable to the levels obtained from stable vector-producing cell lines.

20

25

30

Producer cells can be produced either from packaging cells by introducing into the packaging cell any remaining viral components required for infectious retrovirus production or they can be produced by introduction into a non-packaging cell, such as a 293T cell, of all the components required for infectious retrovirus production.

Producer cells/packaging cells can be of any suitable cell type. Most commonly, mammalian producer cells are used but other cells, such as insect cells are not excluded. Clearly, the producer cells will need to be capable of efficiently translating the env and gag, pol mRNA. Many suitable producer/packaging cell lines are known in the art. The skilled person is also capable of making suitable packaging cell lines by, for example stably introducing a nucleotide construct encoding a packaging component into a cell line.

It is highly desirable to use high-titre virus preparations in both experimental and practical applications. Techniques for increasing viral titre include using a *psi* plus packaging signal as discussed above and concentration of viral stocks. In addition, the use of different envelope proteins, such as the G protein from vesicular-stomatitis virus has improved titres following concentration to 10^9 per ml. However, typically the envelope protein will be chosen such that the viral particle will preferentially infect cells that are infected with the virus which it desired to treat. For example where an HIV vector is being used to treat HIV infection, the env protein used will be the HIV env protein.

Receptors and Retroviral envelope proteins

The endogenous receptor expressed by the producer cell, the expression and/or activity of which it is desired to reduce or inhibit, is able to bind the envelope protein of the infectious retrovirus. Our results indicate that the binding of the envelope protein to the receptor causes a reduction in the retroviral titre produced by the producer cell. Preferably the receptor is an amphotropic receptor and not an ectotropic receptor. A preferred receptor is Pit 2.

The retrovirus envelope protein may be the native envelope protein with respect to the recombinant retrovirus or it may be a different envelope protein if, for example, the retrovirus has been pseudo-typed, the process of producing a retroviral vector in which the envelope protein is not the native envelope of the retrovirus. Certain envelope proteins, such as MLV envelope protein and vesicular stomatitis virus G (VSV-G) protein, pseudotype retroviruses very well. Pseudotyping can be useful for altering the target cell range of the retrovirus. Alternatively, to maintain target cell specificity for target cells

infected with the particular virus it is desired to treat, the envelope protein may be the same as that of the target virus, for example HIV. Preferably the envelope protein is an amphotropic envelope protein and not an ectotropic envelope protein.

- 5 Examples of endogenous receptors and viral envelope proteins that they bind are listed below:

Retroviral Envelope protein	Receptor (human cells)
Simian type D (MPMV, SRV, Baev) Feline endogenous RD114 Avian reticuloendotheliosis viruses	Na ⁺ dependent neutral amino acid transporter; widely expressed in human tissues and cell lines, including haematopoietic cells.
MLV; amphotropic, 4070A, 10A1	Pit 1 and 2
GALV	Pit 1
FeLV-B	Pit 1 (+ Pit 2 for some)
Simian sarcoma associated virus	Pit 1 Na ⁺ dependent phosphate transporters
HIV/SIV	CD4 and co-receptors, e.g. CXCR4, CCR5
Avian sarcoma leukosis viruses subgroup A	small, membrane associated protein. 40 residue cysteine rich motif with homology to low density lipoprotein receptor
Avian sarcoma leukosis viruses subgroups B, D	Cell surface protein resembling receptor for certain cytokines, e.g. tumour necrosis factor

10 Gene products for inhibiting receptor expression

Gene products for use according to the present invention which inhibit expression of an endogenous receptor may do so in several ways. They may interfere with receptor gene transcription, mRNA processing, mRNA stability, mRNA translation, post-translational processing and/or targeting to the relevant cell membrane. It may also be possible to
 15 inhibit the activity of functional receptor by providing a ligand which binds reversibly or irreversibly to the receptor, thus blocking its ability to bind retroviral envelope protein.

The gene product may be expressed in the producer cell by a variety of techniques known to the person skilled in the art. For example a nucleotide sequence encoding the gene product may be introduced into the producer cell. Preferably, the nucleotide sequence encoding the gene product is present in a viral vector, such as a retroviral vector. In particular, it may be possible to include one or more nucleotide sequences encoding gene products in the viral genome used to produce the infectious retrovirus.

It is particularly preferred to use gene products that are capable of effecting the cleavage and/or enzymatic degradation of a target nucleotide sequence, which will generally be a ribonucleotide encoding the receptor. As particular examples, ribozymes, external guide sequences and antisense sequences may be mentioned.

Ribozymes are RNA enzymes which cleave RNA at specific sites. Ribozymes can be engineered so as to be specific for any chosen sequence containing a ribozyme cleavage site. Thus, ribozymes can be engineered which have chosen recognition sites in transcribed viral sequences. By way of an example, ribozymes encoded by the first nucleotide sequence recognise and cleave essential elements of viral genomes required for the production of viral particles, such as packaging components. Thus, for retroviral genomes, such essential elements include the *gag*, *pol* and *env* gene products. A suitable ribozyme capable of recognising at least one of the *gag*, *pol* and *env* gene sequences, or more typically, the RNA sequences transcribed from these genes, is able to bind to and cleave such a sequence. This will reduce or prevent production of the *gag*, *pol* or *env* protein as appropriate and thus reduce or prevent the production of retroviral particles.

Ribozymes come in several forms, including hammerhead, hairpin and hepatitis delta antigenomic ribozymes. Preferred for use herein are hammerhead ribozymes, in part because of their relatively small size, because the sequence requirements for their target cleavage site are minimal and because they have been well characterised. The ribozymes most commonly used in research at present are hammerhead and hairpin ribozymes.

Each individual ribozyme has a motif which recognises and binds to a recognition site in the target RNA. This motif takes the form of one or more "binding arms", generally two

-11-

P006915gb CTH

binding arms. The binding arms in hammerhead ribozymes are the flanking sequences Helix I and Helix III, which flank Helix II. These can be of variable length, usually between 6 to 10 nucleotides each, but can be shorter or longer. The length of the flanking sequences can affect the rate of cleavage. For example, it has been found that reducing the total number of nucleotides in the flanking sequences from 20 to 12 can increase the turnover rate of the ribozyme cleaving a HIV sequence, by 10-fold. A catalytic motif in the ribozyme Helix II in hammerhead ribozymes cleaves the target RNA at a site which is referred to as the cleavage site. Whether or not a ribozyme will cleave any given RNA is determined by the presence or absence of a recognition site for the ribozyme containing an appropriate cleavage site.

Each type of ribozyme recognises its own cleavage site. The hammerhead ribozyme cleavage site has the nucleotide base triplet GUX directly upstream where G is guanine, U is uracil and X is any nucleotide base. Hairpin ribozymes have a cleavage site of BCUGNYR, where B is any nucleotide base other than adenine, N is any nucleotide, Y is cytosine or thymine and R is guanine or adenine. Cleavage by hairpin ribozymes takes places between the G and the N in the cleavage site.

Multiple ribozymes can be included in series in a single vector and can function independently when expressed as a single RNA sequence. A single RNA containing two or more ribozymes having different target recognition sites may be referred to as a multitarget ribozyme. The placement of ribozymes in series has been demonstrated to enhance cleavage.

Antisense technology is well known on the art. There are various mechanisms by which antisense sequences are believed to inhibit gene expression. One mechanism by which antisense sequences are believed to function is the recruitment of the cellular protein RNaseH to the target sequence/antisense construct heteroduplex which results in cleavage and degradation of the heteroduplex. Thus the antisense construct, by contrast to ribozymes, can be said to lead indirectly to cleavage/degradation of the target sequence. Thus according to the present invention, a first nucleotide sequence may encode an antisense RNA that binds to either a gene encoding an essential/packaging component or the RNA transcribed from said gene such that expression of the gene is inhibited, for

example as a result of RNaseH degradation of a resulting heteroduplex. It is not necessary for the antisense construct to encode the entire complementary sequence of the gene encoding an essential/packaging component - a portion may suffice. The skilled person will easily be able to determine how to design a suitable antisense construct.

5

External guide sequences (EGSs) are RNA sequences that bind to a complementary target sequence to form a loop in the target RNA sequence, the overall structure being a substrate for RNaseP-mediated cleavage of the target RNA sequence. The structure that forms when the EGS anneals to the target RNA is very similar to that found in a tRNA precursor. The natural activity of RNaseP can be directed to cleave a target RNA by designing a suitable EGS. The general rules for EGS design are as follows, with reference to the generic EGSs shown in Figure 2:

10

Rules for EGS design in mammalian cells (see Figure 2)

15

Target sequence - All tRNA precursor molecules have a G immediately 3' of the RNaseP cleavage site (i.e. the G forms a base pair with the C at the top of the acceptor stem prior to the ACCA sequence). In addition a U is found 8 nucleotides downstream in all tRNAs. (i.e. G at position 1, U at position 8). A pyrimidine may be preferred 5' of the cut site. No other specific target sequences are generally required.

20

EGS sequence - A 7 nucleotide 'acceptor stem' analogue is optimal (5' hybridising arm). A 4 nucleotide 'D-stem' analogue is preferred (3' hybridising arm). Variation in this length may alter the reaction kinetics. This will be specific to each target site. A consensus 'T-stem and loop' analogue is essential. Minimal 5' and 3' non-pairing sequences are preferred to reduce the potential for undesired folding of the EGS RNA.

25

Deletion of the 'anti-codon stem and loop' analogue may be beneficial. Deletion of the variable loop can also be tolerated *in vitro* but an optimal replacement loop for the deletion of both has not been defined *in vivo*.

30

As with ribozymes, described below, it is preferred to use more than one EGS. Preferably, a plurality of EGSs is employed, together capable of cleaving *gag*, *pol* and *env* RNA of the

native retrovirus at a plurality of sites. Multiple EGSs can be included in series in a single vector and can function independently when expressed as a single RNA sequence. A single RNA containing two or more EGSs having different target recognition sites may be referred to as a multitarget EGS.

5

Further guidance may be obtained by reference to, for example, Werner *et al.* (1997); Werner *et al.* (1998); Ma *et al.* (1998) and Kawa *et al.* (1998).

Therapeutic uses

10

15

The infectious retroviral particles may comprise one or more coding sequences encoding therapeutic products. Therapeutic products include, but are not limited to, cytokines, hormones, antibodies, immunoglobulin fusion proteins, enzymes, immune co-stimulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a single chain antibody, tumour suppresser protein and growth factors. When included, such coding sequences are operatively linked to a suitable promoter.

20

Preferably the viral particles are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Thus, the present invention also provides a pharmaceutical composition for treating an individual, wherein the composition comprises a therapeutically effective amount of the viral particle of the present invention, together with a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The pharmaceutical composition may be for human or animal usage.

25

30

The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

-14-

P006915gb CTH

The pharmaceutical composition may be formulated for parenteral, intramuscular, intravenous, intracranial, subcutaneous, intraocular or transdermal administration.

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The amount of virus administered is typically in the range of from 10^3 to 10^{10} pfu, preferably from 10^5 to 10^8 pfu, more preferably from 10^6 to 10^7 pfu. When injected, typically 1-10 μ l of virus in a pharmaceutically acceptable suitable carrier or diluent is administered.

Where the therapeutic sequence is under the control of an inducible regulatory sequence, it may only be necessary to induce gene expression for the duration of the treatment. Once the condition has been treated, the inducer is removed and expression of the NOI is stopped. This will clearly have clinical advantages. Such a system may, for example, involve administering the antibiotic tetracycline, to activate gene expression via its effect on the tet repressor/VP16 fusion protein.

The invention will now be further described in the Examples which follow, which are intended as an illustration only and do not limit the scope of the invention.

Figure 1 - Sequence and secondary structure of riboram.

Figure 2 — design of external guide sequences.

EXAMPLES

- 5 **Example 1 — Envelope is not limiting in ecotropic particles compared to amphotropic, rabies-pseudotyped and GALV-pseudotyped particles**

We investigated the limiting components in retroviral production using a transient transfection method. The genetic elements required to produce retroviral vectors capable of transducing cells were separated into three expression plasmids: one carrying the *gag-pol* gene, one carrying the envelope gene and the third bearing the packaging signal and *lacZ* gene that are flanked by the long terminal repeats (LTRs). Virions are produced when all three plasmids are transfected into 293T cells (Soneoka *et al.*, 1995). Raising the amount of one plasmid with respect to the other two, we measured the viral titres and compared them to the titres produced when equal amounts of all three plasmids were used.

Table 1.

Amounts of plasmids (μg) ^a			Titres (i.f.u./ml) ^b			
<i>gag-pol</i>	Genome	Envelope	Ecotropic ^c	Amphotropic ^c	Rabies ^d	GALV ^e
0.1	0.1	0.1	5.0×10^3	6.5×10^3	1.3×10^3	1.5×10^3
1.0	0.1	0.1	5.2×10^3	1.6×10^3	4.4×10^2	5.0×10^2
0.1	1.0	0.1	1.2×10^5	4.1×10^4	2.2×10^4	1.0×10^4
0.1	0.1	1.0	8.0×10^3	1.9×10^4	9.0×10^3	8.1×10^3
1.0	1.0	1.0	2.5×10^5	3.5×10^5	1.7×10^5	1.5×10^5
0.1	1.0	1.0	1.4×10^5	1.6×10^5	2.5×10^4	3.0×10^4

- 20 ^aDifferent amounts of plasmids were used to transfect 293T cells in 6 cm dishes using FuGene6 transfection reagent (Boehringer Mannheim). ^bViral titres were measured as the number of *lacZ* forming units (i.f.u.) per ml as observed by X-gal staining ^cTitrated on NIH3T3 cells, ^dTitrated on BHK21 cells, ^eTitrated on HT1080 cells.

It was found that the amphotropic, rabies and GALV envelopes can raise the viral titres but not the ecotropic envelope (Table 1). This indicated that the amphotropic, rabies and GALV envelopes were limiting during viral production but not the ecotropic envelope. We deduced that the envelope is only limiting when its receptors are found in the 293T producer cells. A logical explanation for this observation is that the interaction between the envelope and its receptors limits the pool of envelope available for incorporation into virions. This interaction has been reported in replication competent wild-type retroviruses (Hunter, 1997).

Example 2 – Down-regulation of the amphotropic receptor *pit2* in producer cells

Ribozyme design and construction

The mRNA of the amphotropic receptor *pit2* is folded using the RNA draw programme. From the secondary structure of the RNA, two ribozymes are designed to target exposed regions while a third is designed to target the envelope-binding site. The order in which the three ribozymes is put together in a single construct is decided by folding the constructs using the RNA draw programme and selecting the one which has the least stable secondary structure (Figure 1). This is to ensure that the binding of the ribozymes to the mRNA of *pit2* is not obstructed by secondary structures within the ribozyme construct.

Two oligonucleotides corresponding to the sequence of the ribozymes and its complementary sequence flanked by *Bam*HI and *Eco*RI restriction sites are synthesised. They are annealed and cloned into pBluescript. The resulting plasmid is designated pRiboram.

In vitro testing

The pRiboram is transcribed *in vitro* and the ribozyme is used to cleave the mRNA of *pit2*, which has also been transcribed *in vitro*. The cleaved products are detected on an agarose gel, indicating the ribozyme can function *in vitro*.

-17-

P006915gb CTH

In vivo testing

The ribozyme construct is then sub-cloned from pRiboram into the *BglIII* - *EcoRI* site of pSA91, which is a mammalian expression vector driven by the hCMV promoter (Reference). This plasmid is designated pCRiboram. pCRiboram was co-transfected with different combinations of amounts of *gag-pol*, *env* and genome expression plasmids into 293T cells. It is found that amphotropic envelope is no longer limiting.

Other aspects of the present invention are presented in attached Figures 3 to 14.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

References

5 Gilbert, J. M., P. Bates and H. E. Varmus (1994). "The receptor for the subgroup A avian leukosis-sarcoma viruses binds to subgroup A but not to subgroup C envelope glycoprotein." *J. Virol.* **68**: 5623 - 5628.

Hunter, E. (1997). Synthesis, assembly, and processing of viral proteins. Retroviruses. J. M. Coffin, S. H. Hughes and H. E. Varmus, Cold Spring Harbor Press: 263 - 334.

10 Jabbar, M. A. (1995). "The human immunodeficiency virus type 1 Vpu protein: Roles in virus release and CD4 downregulation." *Curr. Top. Microbiol. Immunol.* **193**: 107 - 120.

Kawa *et al.*, 1998, *RNA* **4**: 1397-1406.

15 Ma *et al.*, 1998, *Antisense and Nucleic Acid Drug Development* **8**: 415-426.

Miller, A. D. (1997). Development and applications of retroviral vectors. Retroviruses. J. M. Coffin, S. H. Hughes and H. E. Varmus, Cold Spring Harbor Press: 437 - 473.

20 Soneoka, Y., P. M. Cannon, E. E. Ramsdale, J. C. Griffiths, G. Romano, S. M. Kingsman, and A. J. Kingsman. (1995). A transient three plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res.* **23**:628-33.

Werner *et al.*, 1997, *Nucleic Acids Symposium Series* No. 36: 19-21.

25 Werner *et al.*, 1998, *RNA* **4**: 847-855.

CLAIMS

1. A method for enhancing the production of an infectious retrovirus comprising an envelope polypeptide in a producer cell which method comprises inhibiting the expression or activity in the producer cell of an endogenous receptor which is capable of binding to the envelope polypeptide of said retroviruses.
2. A method according to claim 1 wherein the receptor is selected from Pit1, Pit2 and CD4 and its coreceptors.
3. A method according to claim 1 or 2 wherein the envelope polypeptide is an amphotropic envelope polypeptide.
4. A method according to any one of claims 1 to 3 wherein expression of the receptor is inhibited by expressing in the producer cell a gene product capable of binding to and effecting the cleavage, directly or indirectly, of a nucleotide sequence encoding the receptor, or a transcription product thereof.
5. A method according to claim 4 wherein the gene product is selected from a ribozyme, an anti-sense ribonucleic acid and an external guide sequence.
6. A method according to claim 4 wherein the gene product is expressed by a viral vector.
7. A method according to claim 6 wherein the viral vector is a retroviral vector.
8. A method according to any one of the preceding claims wherein the retrovirus is a lentivirus.
9. A method according to any one of the preceding claims which further comprises isolating the infectious retrovirus produced by the producer cell.

-20-

P006915gb CTH

10. A composition comprising an infectious retrovirus obtained by the method of claim 9.

11. A composition according to claim 10 for use in therapy.

12. A method for producing a pharmaceutical composition which method comprises isolating an infectious retrovirus produced by the producer cell according to the method of any one of claims 1 to 8 and admixing the isolated infectious retrovirus with a pharmaceutically acceptable carrier, diluent or excipient.

13. A nucleic acid comprising a nucleotide sequence encoding a ribozyme capable of binding to and effecting the cleavage of an RNA encoding a *pit2* receptor.

14. A nucleic acid according to claim 13 comprising a nucleotide sequence as shown in Figure 1 or a variant thereof capable of binding to and effecting the cleavage of an RNA encoding a *pit2* receptor.

THIS PAGE BLANK (USPTO)

-21-

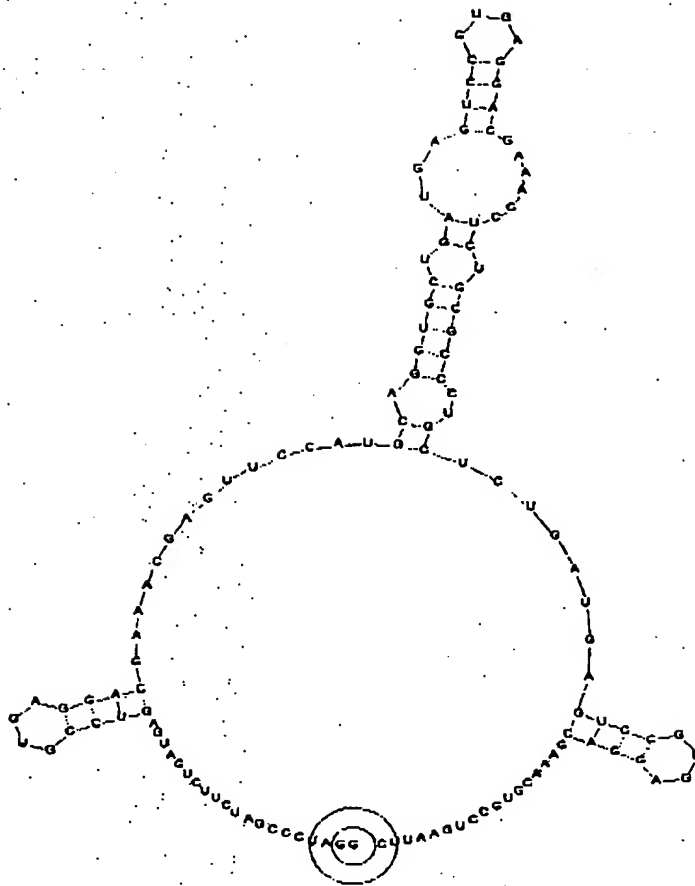
P006915gb CTH

ABSTRACT**IMPROVED RETROVIRAL PRODUCTION**

- 5 A method is provided for enhancing the production of an infectious retrovirus comprising an envelope polypeptide in a producer cell which method comprises inhibiting the expression or activity in the producer cell of an endogenous receptor which is capable of binding to the envelope polypeptide of said retroviruses.

THIS PAGE BLANK (USPTO)

Figure 1

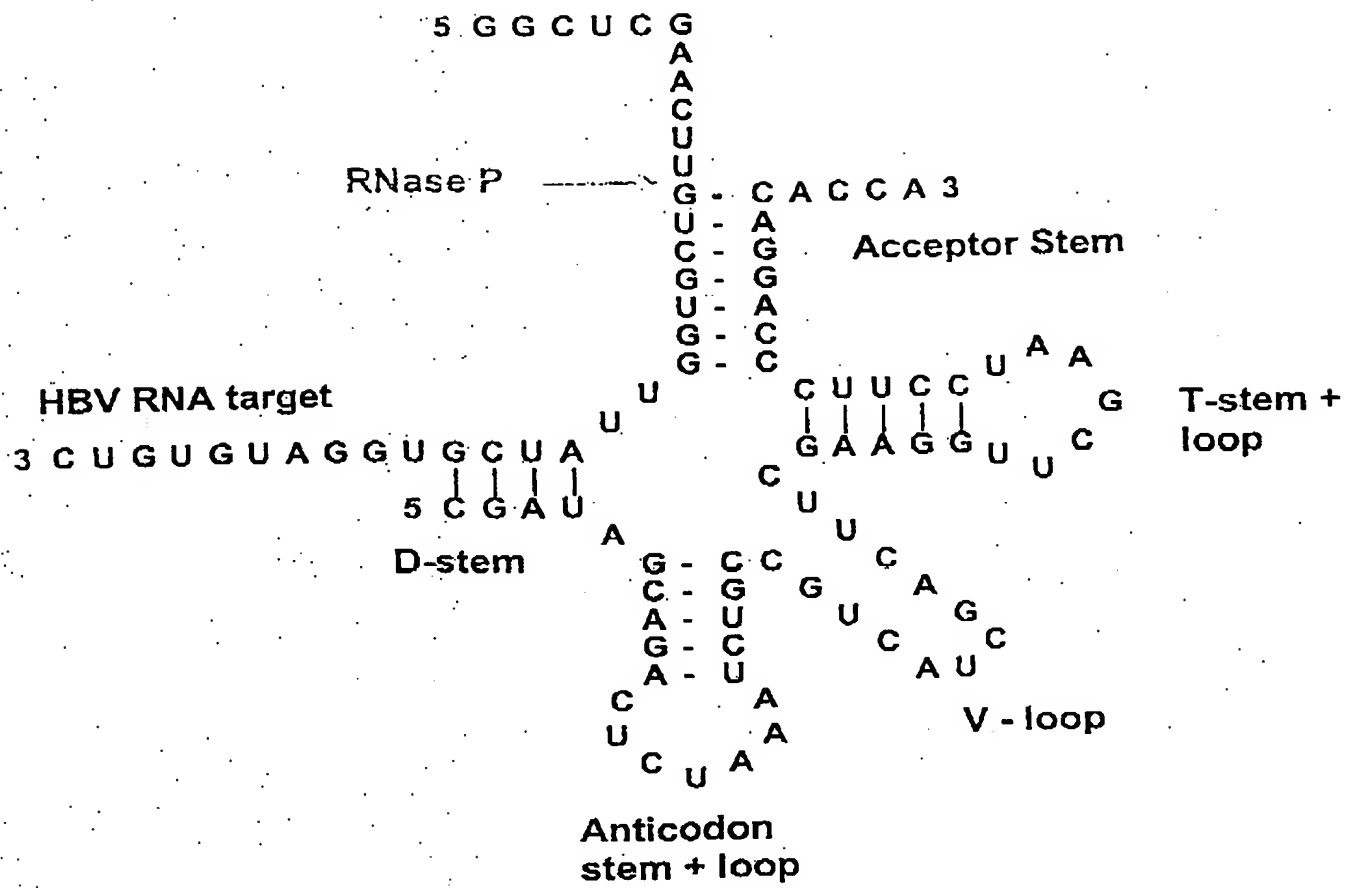


5'-GGAUCCCGAUCUUCUGAUGAGUCCGUGAGGACGAAACGAGUUCCAUGCAG
GUGCUGAUGAGUCCGUGAGGACGAAACCUCUGCGCCUGCUCUGAUGAGUC
CGUGAGGACGAAACGUGCCUGAAUUC-3'

1/15

THIS PAGE BLANK (USPTO)

Figure 2A



EGS Based on Tyrosyl t-RNA

THIS PAGE BLANK (USPTO)

THIS PAGE BLANK (USPTO)

fig 3

1

Introduction

Retroviruses are presented with a paradox in their life-cycle: the receptors on the host cell which permit them to enter and infect the host cell also interact with newly synthesized envelope proteins in the infected cell and hinder viral production. In complex retroviruses like HIV, the problem is solved through down-regulation of the receptor by the *vpu* gene product (Jabbar, 1995). In other retroviral systems, mechanisms to prevent receptor-envelope interaction have not been described (Swanstrom and Wills, 1997).

A transient transfection system had been developed to produce retroviral vectors from viral components that were segregated into 3 plasmids (*gag/gag-pol*, *env* and *genome*)(Soneoka et al, 1995). We have shown that under conditions where none of the viral components were saturating, the viral envelope was limiting when its cognate receptor was present on the producer cell.

4/15

THIS PAGE BLANK (USPTO)

2

Transient three-plasmid expression system for expression of retroviral vectors

Soneoka *et. al.* 1995. Nucleic Acids Res 23(4): 628-33

gag-pol plasmid
genome plasmid
env plasmid



Transfection of 293T Cells



Replace with NaBu-containing media
12 hrs post-transfection



Replace with complete media
after NaBu treatment for
12 hours



Harvest virus and transduce
target cells.



Figure 4

5/15

THIS PAGE BLANK (USPTO)

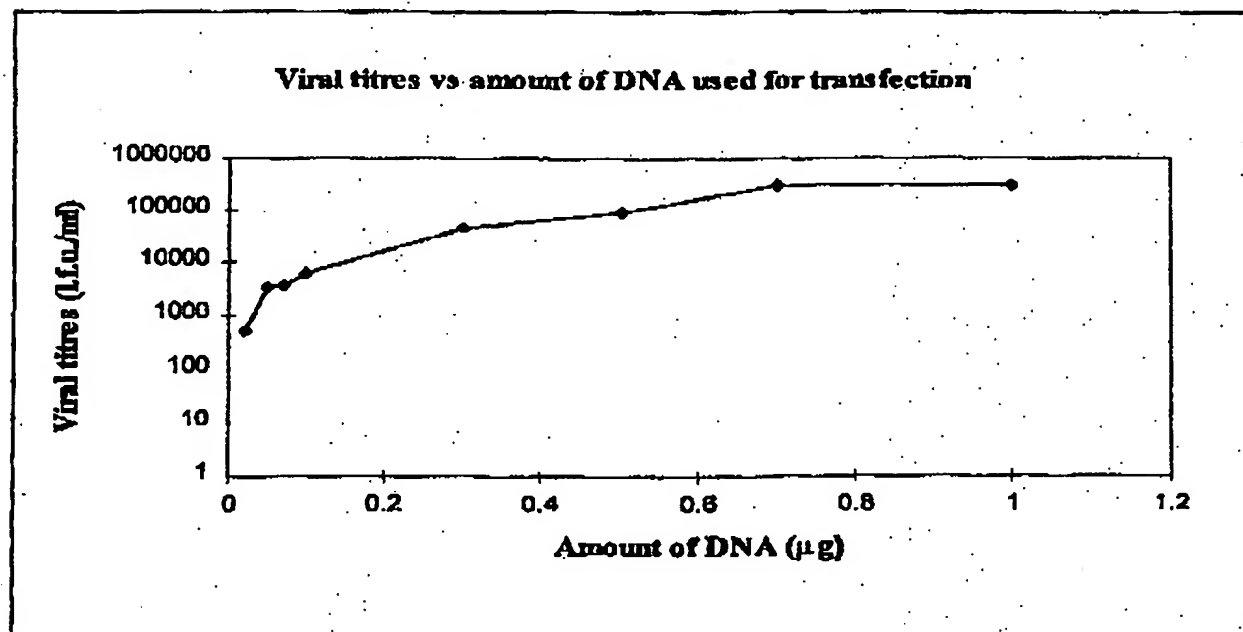
Fig 5

3

Determination of the conditions under which none of the components are saturating

None of the components are saturating at 0.1 μg of each plasmid.

0.1 μg was therefore used as a starting point from which each component was raised.



6/15

THIS PAGE BLANK (USPTO)

fig 6

4

Effect of each component on the viral titres

Table 1: The number of infectious particles was measured by counting the number of transduced cells observed by x-gal staining.

Amounts of plasmids used in transfection (μ g)			Titres (i.f.u./ml)
<i>Gag/gag-pol</i>	Genome	Amphotropic envelope	
0.1	0.1	0.1	$6.5 \pm 0.9 \times 10^3$
1	0.1	0.1	$1.6 \pm 0 \times 10^3$
0.1	1	0.1	$4.1 \pm 0.1 \times 10^4$
0.1	0.1	1	$1.9 \pm 0.4 \times 10^4$
1	1	1	$3.5 \pm 0.5 \times 10^3$
0.1	1	1	$1.6 \pm 0.6 \times 10^3$

• 10 fold more *gag/gag-pol* reduced titres. Therefore, *gag/gag-pol* had a negative effect on titres.

• Genome and *env* were limiting since titres could be raised by increasing these during transfection.

• Negative effect of *gag/gag-pol* was observed only under limiting conditions of *env* and genome.

7/15

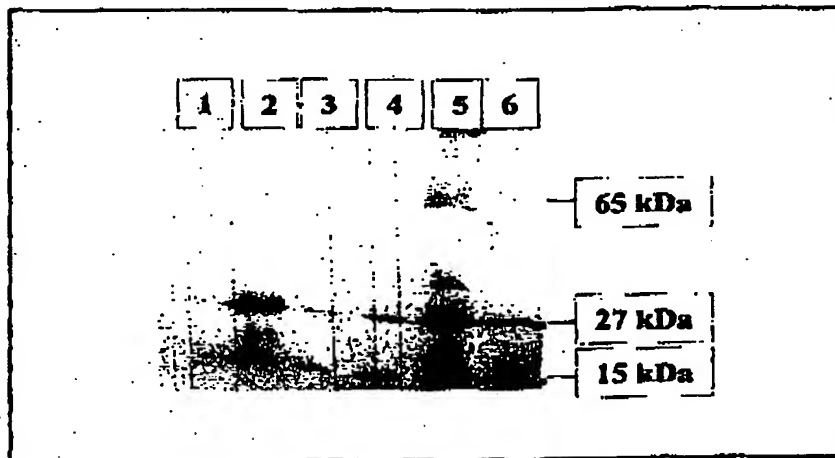
THIS PAGE BLANK (USPTO)

fig 7

5

Western blot analysis of viral supernatants

- To investigate the total number of particles produced, a western blot analysis was performed on the viral stocks produced in table 1.
- More particles were produced when more *gag/gag-pol* component (samples 2 & 5) was used.
- A large proportion of defective particles must be present in sample 2 since it had low titres despite having more particles.



Western analysis of viral supernatants using Anti-p15 (*gag*). 300 μ l of viral supernatant was pelleted and loaded in each lane. Lane 1: 0.1 μ g of all three plasmids; Lane 2: 1 μ g of *gag-pol*, 0.1 μ g of genome and *env*; Lane 3: 1 μ g of genome, 0.1 μ g of *gag-pol* and *env*; Lane 4: 1 μ g of *env*, 0.1 μ g of *gag-pol* and genome; Lane 5: 1 μ g of all three plasmids; Lane 6: 0.1 μ g of *gag-pol*, 1 μ g of genome and *env*.

THIS PAGE BLANK (USPTO)

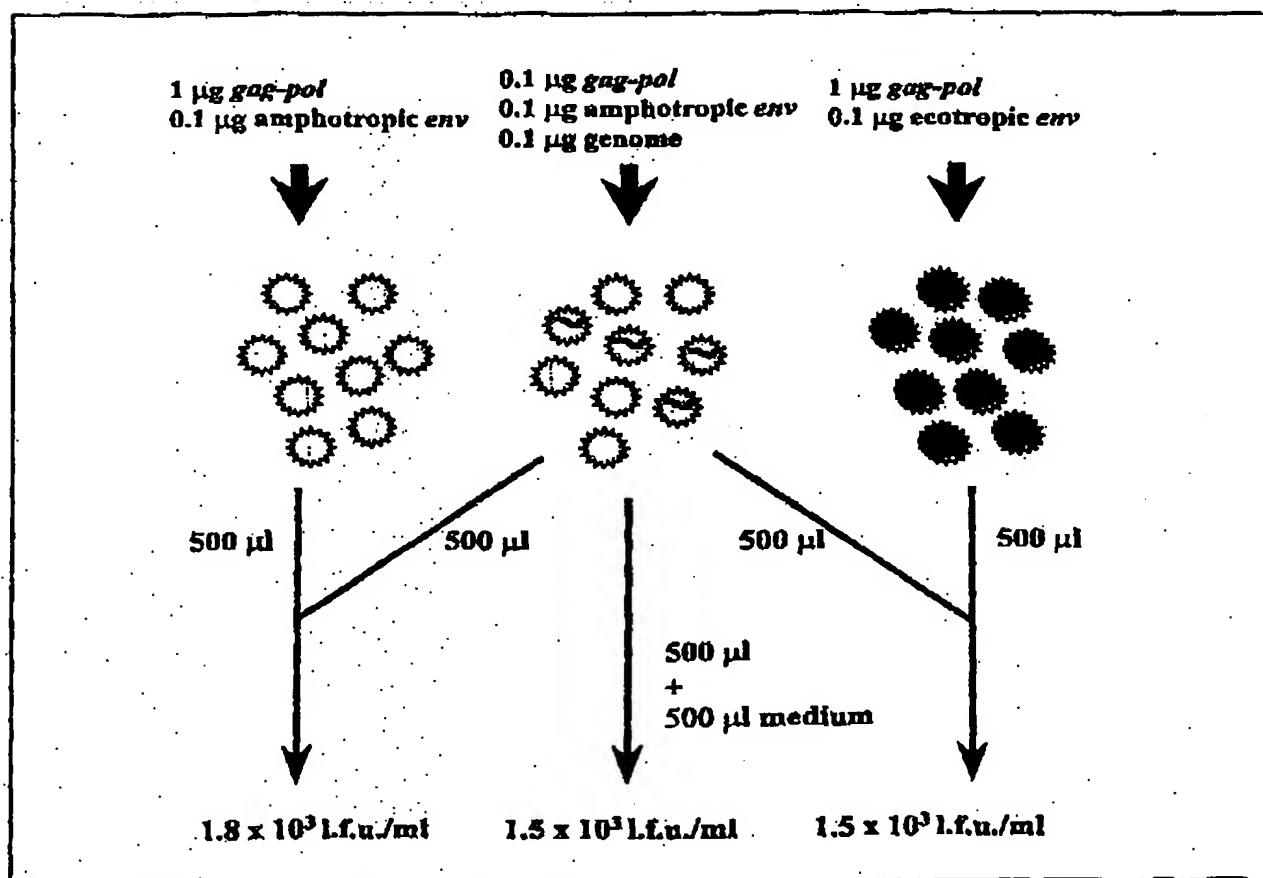
fig 8

6

Is the negative effect of *gag-pol* on viral titres due to interference by defective particles?

• One explanation for the negative effect of *gag/gag-pol* on viral titres was that the defective particles were interfering with the binding of infectious particles.

• To test this hypothesis, the following experiment was performed:



- No decrease in titres was observed when either amphotropic or ecotropic empty particles were present in the viral stocks.

NO

- The decrease in titres was not due to obstruction of receptors by enveloped defective particles.

9/15

THIS PAGE BLANK (USPTO)

fig 9

7

Elimination of the negative effect of *gag-pol*

•The negative effect of *gag/gag-pol* was shown not to be due to an extracellular event. We therefore focused our attention on the intracellular events during viral production.

•To investigate if the negative effect of *gag-pol* could be cancelled by *env* or genome, the following sets of transfections were performed:

Amounts of plasmids (μg)			Viral titres (i.f.u./ml)
<i>Gag/gag-pol</i>	pHT111	Amphotropic envelope	
0.1	0.1	0.1	$6.5 \pm 0.9 \times 10^3$
1	0.1	0.1	$1.6 \pm 0 \times 10^3$
0.1	1	0.1	$4.1 \pm 0.1 \times 10^3$
1	1	0.1	$1.3 \pm 0.1 \times 10^3$
0.1	0.1	1	$1.9 \pm 0.4 \times 10^3$
1	0.1	1	$1.3 \pm 0.1 \times 10^3$

•Titres do not decrease in the presence of excess *gag/gag-pol* when *env* or genome is not limiting.

•The negative effects of *gag/gag-pol* can be cancelled by *env* or genome.

10/15

THIS PAGE BLANK (USPTO)

fig 10

8

Effects of different envelopes on viral titres

Since the amphotropic envelope was found to abolish the negative effect of *gag/gag-pol* on titres, an investigation was conducted to study the effects of other envelopes on viral titres:

Amounts of plasmids (μg) ^a			Titres (l.f.u./ml) ^b			
<i>Gag/gag-pol</i>	Genome	Envelope	Ecotropic ^c	VSV-G ^c	Rabies ^d	GALV ^e
0.1	0.1	0.1	3.0×10^3	1.9×10^3	1.3×10^3	1.5×10^3
1.0	0.1	0.1	5.2×10^3	0.9×10^3	4.4×10^2	5.0×10^2
0.1	1.0	0.1	1.2×10^3	2.6×10^3	2.2×10^3	1.0×10^4
0.1	0.1	1.0	8.0×10^3	3.2×10^3	9.0×10^3	8.1×10^3
1.0	1.0	1.0	2.5×10^3	7.0×10^4	1.7×10^3	1.5×10^4
0.1	1.0	1.0	1.4×10^3	2.3×10^4	2.5×10^4	3.0×10^4

^aDifferent amounts of plasmids were used to transfect 293T cells in 6 cm dishes using FuGene6 transfection reagent (Boehringer Mannheim).

^bViral titres were measured as the number of *lacZ* forming units (l.f.u.) per ml as observed by X-gal staining

^cTitrated on NIH3T3 cells

^dTitrated on BHK21 cells

^eTitrated on HT1080 cells

•Ecotropic and VSV-G envelopes:

env is saturating and there is no negative effect of *gag/gag-pol* on titres

•GALV and Rabies envelopes:

env is not saturating and there is a negative effect of *gag/gag-pol* on titres

•Therefore, the negative effect of *gag/gag-pol* is envelope dependent.

•These data imply that the effective concentration of *env* available for particle formation is less when GALV and Rabies are used compared to VSV-G and ecotropic. The most likely explanation is that GALV and Rabies envelopes are sequestered. This results in release of naked particles which are non-infectious, therefore effectively reducing the titres.

11/15

THIS PAGE BLANK (USPTO)

fig 11

9

The identification of receptors in 293T producer cells

•To test the hypothesis that interaction with receptors limit availability of envelopes for incorporation, the presence of receptors for the different envelopes in 293T cells was investigated indirectly by determination of titres using different envelopes.

•293T cells were transduced with MoMLV based vectors pseudotyped with different envelopes:

Type of envelope	Titres (I.f.u./ml)
Amphotropic	4.3×10^5
Ecotropic	0
Rabies-G	7.0×10^4
VSV-G	4.0×10^5

293T cells had been shown to be transduced by GALV pseudotyped particles (Eglitis et al, 1995).

Conclusions:

293T producer cells express receptors for the amphotropic envelope, rabies-G, GALV and VSV-G but not receptors for the ecotropic envelope

12/15

THIS PAGE BLANK (USPTO)

Fig 12

10

Summary

Envelope	Receptors expressed in 293T cells?	Negative effect of gag/gag-pol on titres?	Is envelope limiting?
Amphotropic	Yes	Yes	Yes
Ecotropic	No	No	No
GALV	Yes	Yes	Yes
Rabies-G	Yes	Yes	Yes
VSV-G	Yes	No	No

- The negative effect of *gag/gag-pol* on titres was observed only when envelope was not saturating.
- The envelope seemed to be limiting when its cognate receptor was expressed in the producer cell (293T) and not when it was absent.
- These data support the hypothesis that interaction with receptors can limit the availability of functional envelope (figure 13).
- VSV-G does not seem to conform with the hypothesis. The receptor of VSV-G is phosphatidylserine, a membrane phospholipid (Pal et al, 1987), which might not interact with the envelope on the membrane.

13/15

THIS PAGE BLANK (USPTO)

fig 13

11

Hypothesis for envelope-dependent negative effect of *gag/gag-pol* on viral titres

Based on the results obtained, the following hypothesis is proposed:

Legend

- Envelope
- ! Receptor
- ⊙ Genome-containing core
- Empty core
- ⊙ Bald genome-containing particle
- ⊙ Bald empty particle
- ⊙ Envelope and genome-containing particle
- ⊙ Envelope containing empty particle

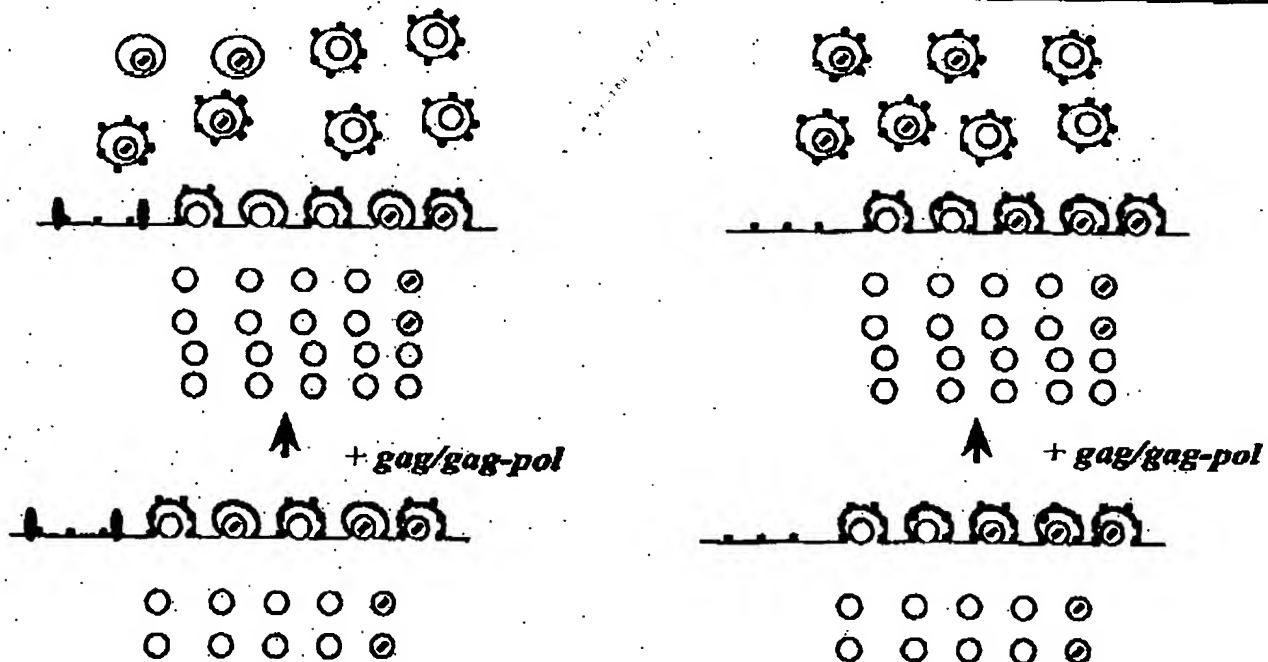


Figure 13

Receptors interacted with envelope and limited the pool of envelope available for incorporation into virions. Therefore, envelope was limiting.

Excess *gag/gag-pol* produced more empty cores which competed with genome-containing cores for envelope during assembly. Hence, there was a decrease in envelope and genome containing particles, manifested as a decrease in titres.

THIS PAGE BLANK (USPTO)

Fig 14

12

References for Figures 3 - 13.

Eglitis M.A., R.D. Schneiderman, P.M. Rice and M.V. Eiden. (1995). Evaluation of retroviral vectors based on the gibbon ape leukaemia virus. Gene Ther. 2(7): 486 - 92.

Jabbar, M. A. (1995). "The human immunodeficiency virus type 1 Vpu protein: Roles in virus release and CD4 downregulation." Curr. Top. Microbiol. Immunol. 193: 107 - 120.

Pal, R., Y. Barenholz and R.R. Wagner. (1987). Vesicular stomatitis virus membrane proteins and their interactions with lipid bilayers. Biochimica et Biophysica Acta. 906: 175 - 193.

Soncoka, Y., P.M. Cannon, E.E. Ramsdale, J.C. Griffiths, G. Romano, S.M. Kingsman and A.J. Kingsman. (1995). A transient three-plasmid expression system for the production of high titre retroviral vectors. Nucleic Acids Res. 23: 628 - 633

Swanstrom, R. and J.W. Wills. (1997). Synthesis, Assembly and Processing of Viral Proteins. In: Retroviruses. CSHL Press

15/15

THIS PAGE BLANK (USPTO)

~~BEST AVAILABLE COPY~~